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Presynaptic effects of octopamine, serotonin, and cocktails of the two modulators on neuromuscular transmission in crustaceans

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Abstract The effect of the biogenic amines octopamine and serotonin, and of both amines combined (cocktails) on transmitter release at neuromuscular junctions of two crustaceans was studied. octopamine (10^{-8} mol l^{-1} to 10^{-6} mol l^{-1}) either enhanced or decreased evoked transmitter release through presynaptic effects. The results were identical for the slow and the fast excitor in the closer muscle of the crab, and for the excitor in the opener muscle of the crayfish. Application of serotonin always resulted in a strong increase of release. However, this potentiating effect of serotonin was reduced in strength by subsequent application of cocktails consisting of serotonin and octopamine. In all experiments, a cocktail of serotonin and octopamine was less effective than serotonin alone. The decrease in the mean quantal content *m* by octopamine was due to a reduction of the probability of release *p*. Since both amines are synthesized in the central nervous system and are released from neurohaemal organs into the haemolymph bathing the neuromuscular junctions, the results suggest that the two amines, when present together, modulate transmitter release in an antagonistic way, and that the level of the two determines synaptic efficacy.

Key words Crayfish · Crab · Transmitter release · Biogenic amine · Modulation

Abbreviations *5-HT* serotonin · *EPSC* excitatory postsynaptic current · *EPSP* excitatory postsynaptic potential · *FCE* fast closer excitor · *OE* opener excitor · *SCE* slow closer excitor · *NMJ* neuromuscular junction · *OA* octopamine

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Introduction

Neuromodulators such as biogenic amines or peptides are well recognized from work on invertebrates to vertebrates as substances that can change the efficacy of classical neurotransmitters in many physiological processes and even influence behavior. In crustaceans, the amine modulators serotonin (5-HT) and octopamine (OA) are of particular interest since they are known to be involved in the modulation of a very broad range of activities such as regulation of heart beat (Florey and Rathmayer 1978), setting the responsiveness of neurons in the central nervous system (Glanzmann and Krasne 1983; Flamm and Harris-Warrick 1986; Mulloney et al. 1987; Ma et al. 1992; Barthe et al. 1993; Johnson et al. 1993; Ma and Weiger 1993; Strawn et al. 2000) and of sensory receptor cells (Pasztor and MacMillan 1990; Rossi-Durand 1993), excitation-contraction coupling (Fischer and Florey 1983, 1987; Goy and Kravitz 1989), and synaptic transmission at neuromuscular junctions (Dudel 1965; Kravitz et al. 1980; Enyeart 1981; Glusman and Kravitz 1982; Fischer and Florey 1983; Harris-Warrick and Kravitz 1984; Dixon and Atwood 1985; Goy and Kravitz 1989; Delaney et al. 1991; Qian and Delaney 1997; Jorge-Rivera et al. 1998; Vyshedskiy et al. 1998; Wang and Zucker 1998; Beaumont and Zucker 2000). Even behavioral reactions such as escape reflexes (Yeh et al. 1996, 1997), postural display and aggressiveness (Livingston et al. 1980; Harris-Warrick and Kravitz 1984; Harris-Warrick 1985; Beltz and Kravitz 1986; Antonsen and Paul 1997; Huber et al. 1997; Huber and Delgado 1998; Kravitz 2000; Sneddon et al. 2000) are modulated by these two amines. Immunocytochemical studies revealed neurons which contain 5-HT or OA and release these substances in the central nervous system and/or in

neurohaemal organs (Evans et al. 1976; Konishi and Kravitz 1978; Sullivan 1978; Beltz and Kravitz 1983; Schneider et al. 1993, 1996; Thompson et al. 1994; Beltz 1999; Tierney et al. 1999). Since crustaceans have an open circulatory system, modulators released from neurohaemal organs will indirectly reach the neuromuscular junctions.

In crustaceans, mechanisms of action of neuromodulators can be investigated directly at synaptic sites by use of focal macropatch recordings at identified neuromuscular junctions (Dudel 1981; Cooper et al. 1995; Kreissl et al. 1999). Earlier studies at neuromuscular junctions in crustaceans have shown that 5-HT or OA enhances the amplitudes of excitatory and inhibitory junctional potentials in most of the preparations studied (Dudel 1965; Florey and Rathmayer 1978; Kravitz et al. 1980; Enyeart 1981; Jacobs and Atwood 1981; Glusman and Kravitz 1982; Breen and Atwood 1983; Fischer and Florey 1983; Dixon and Atwood 1985; Delaney et al. 1991; Wang and Zucker 1998). The potentiating effects of 5-HT are produced upon binding to a G_S protein-coupled receptor through IP_3- and cAMP-dependent pathways (Batelle and Kravitz 1978; Enyeart 1981; Dixon and Atwood 1989a, 1989b; Goy and Kravitz 1989; Beaumont and Zucker 2000). A recent study has demonstrated direct actions of 5-HT on hyperpolarization-activated cation channels in the presynaptic axon terminals (Beaumont and Zucker 2000). The enhancement of transmitter release by 5-HT can involve two distinct mechanisms: an increase in both the probability of vesicle release (Wang and Zucker 1998; Southard et al. 2000), and, in inhibitory terminals, acceleration of the kinetics of release (Vyshedskiy et al. 1998). Comparable studies of OA mechanisms have not been carried out.

Previous studies addressed the roles of 5-HT and OA as single substances, but as these modulators should occur simultaneously in the haemolymph, we investigated their neuromuscular effects with sequential exposure and in combined application as cocktails in order to better understand their actions within the animal. To our surprise we found that OA, although in some preparations it showed the well known enhancement of transmitter release, actually reduced transmitter output in a large number of junctions. This is in accord with a recent finding that at larval neuromuscular junctions of *Drosophila* OA also reduces transmitter release (Nishikawa and Kikodoro 1999). In all other insect preparations, an increase of excitatory postsynaptic potential (EPSP) amplitudes by OA was observed (for review see Roeder 1999). For 5-HT we always found the well-known potentiation of transmitter release, but this effect was invariably reduced in the presence of OA. This new finding indicates that the two amines, when present together, can antagonistically modulate transmitter release at neuromuscular junctions of crayfish and crab.

Materials and methods

Animals

Crabs, *Eriphia spinifrons*, were collected in the bay of Naples, Italy, and kept in artificial seawater at 16°C. Electrophysiological studies were performed exclusively on identified slow-contracting type I fibers nos. 2 and 3 (Rathmayer and Maier 1987) of the closer muscles of the first three pairs of walking legs. The legs were obtained by inducing autotomy. The slow and the fast closer excitors (SCE and FCE, respectively) were used for stimulation. Preparation, composition of the muscle of different fiber types, and methods for isolation and stimulation of individual motor axon in the meropodite have been described previously (Rathmayer and Erxleben 1983).

Crayfish, *Procambarus clarkii* (6–10 cm body length), were obtained from Atchafalaya Biological Supply (Raceland, La., USA). They were kept singly in individual freshwater tanks and fed fish food pellets weekly until the time of experimentation. Only male crayfish in the intermolt stage were used for experimentation.

Solutions

The saline for the experiments with the crab had a composition of (mmol l^{-1}): 490 NaCl, 8 KCl, 10 $CaCl_2$, 12 $MgCl_2$, and 10 HEPES at pH 7.4, for crayfish 205 NaCl, 5.3 KCl, 13.5 $CaCl_2$, 2.45 $MgCl_2$, and 0.5 HEPES at pH 7.4. The amines were dissolved in distilled water at 1 mmol l^{-1} concentration and stored at –20°C. Stock solution aliquots were diluted in saline prior to experiments. The amine-containing solutions were applied to the muscle directly at the recording sites through a gravity superfusion system (experiments with the crab) or to the bath through a pipette (crayfish preparations). After each change of solutions, a 5 min interval was allowed for equilibration before resuming the recording. OA and 5-HT were obtained from Sigma. All experiments were carried out at controlled room temperature of 19–20°C.

Postsynaptic potentials

Intracellular recordings were obtained in the opener muscle of the crayfish by standard techniques. Signals were recorded to a VHS tape (Vetter, 400) and on-line to a PowerMac 9500 via a MacLab/4s interface (ADInstruments, Australia). All events were measured and calibrated with the MacLab Chart software version 3.5.6 (ADInstruments) with an acquisition rate set at 10 kHz.

Postsynaptic currents

In the crab preparations, excitatory axons were individually stimulated, the SCE with twin pulses at 30 Hz with a low repetition rate (0.5 Hz) to avoid facilitation of the response to the first stimulus, the FCE with single pulses at 0.5 Hz. Evoked excitatory postsynaptic currents (EPSCs) were recorded focally from individual release varicosities using macropatch electrodes (Dudel 1981) with open diameters of about 10 μm (electrode resistance 0.1–0.3 MOhm). The macropatch electrode is specific for current recording within the region of the electrode lumen. The patch electrode was filled with the saline of the bathing medium. Optimal release sites were identified by scanning the fiber with the electrode for sites which produced fast-rising EPSCs and single quanta responses with an amplitude of about 500 pA. The seal resistance of the macropatch electrode was monitored by applying a test current pulse through the electrode. Only preparations in which seal resistance did not change by more than 5% over the period of the experiment were used for further analyses.

Synaptic currents from the crayfish opener muscle were recorded with a focal macropatch electrode. A loose patch was made

by lightly placing a 10- to 20-m-diameter, fire-polished, glass electrode directly over a single, spatially isolated varicosity along a vital dye-visualized nerve terminal. To visualize the nerve terminals, the preparations were fluorescently stained for 2–5 min with 2–5 μmol l^{-1} 4-[4-(diethylamino) styryl]-*N*-methylpyridinium iodide (4-Di-2-Asp; Molecular Probes, Eugene, Ore., USA) in saline, followed by washing. The synaptic transmission remains unaltered by this dye (Cooper et al. 1996). The seal resistance was in the range of 100 KOhm to 1 MOhm. Since the seal can easily be lost if the muscle contracts under the electrode, stimulation in the crayfish was restricted to 1 Hz. In this preparation, the determination of quantal release is possible by determining the area of the recorded current which is a measure of charge. It should be noted that the time of peak-evoked events varies for the crayfish opener neuromuscular junction (NMJ) because of latency jitter of release, so the measurements of peak amplitude are not as reliable as the charge measure when multiple events occur. This is not the case at the crab slow closer terminals on the fiber type used. The change in the charge measures before and after application of 5-HT or OA to the nerve terminals were calculated.

Statistical significance was tested by employing Student's *t*-test. Data are presented as mean ± standard error (SEM).

Quantal analysis

Several methods were employed to quantify quantal release parameters. When quantal content was low (e.g., with the first EPSC in the twin pulse stimulation of SCE), the mean quantal content (*m*) of EPSCs was determined directly by counting the number of zero releases (failures) and, in the case of release, the individual quanta and relating them to the number of trials given. The number of putative release sites (*n*) and the mean probability for release at the recorded site (*p*) were calculated by adopting binomial statistics by two commonly used procedures (Zucker 1973; Cooper et al. 1995). Usually, 200–300 samples were analyzed. When quantal content of the EPSCs was high, which was usually the case for the second of the pulses to SCE and for all EPSCs following FCE stimulation, counting the number of quanta released by each impulse was no longer feasible. Therefore, with 0.5-Hz stimulation, 200–300 EPSCs were averaged electronically.

The EPSCs obtained in the crayfish preparations were also used to determine *m*, *n*, and *p* (Zucker 1973; Cooper et al. 1995). In each synaptic current recording, a stimulation artifact and the nerve terminal potential can be seen which allows to determine if a stimulation failed or if synaptic release failed in spite of nerve terminal depolarization. Mean quantal content (*m*) was determined by direct counts. Direct counts of the evoked quantal events allowed the distribution of release to be further characterized into the type of distribution in order to estimate *n* and *p*. The data sets were tested for a best-fit approximation based on assumptions discussed in earlier reports (Zucker 1973; Cooper et al. 1995). Binomial distributions are known to represent the quantal nature of release in crayfish neuromuscular junctions. The -squared statistic and a modified Akaike information criterion (AIC) were used to estimate the distribution that best fits the observed distribution of events. In addition, in the case of binomial distributions a bootstrapping procedure was used to estimate *n* and *p* (Cooper et al. 1995). Since the exposure to 5-HT and OA produced gradual changes in all the quantal parameters, sample sets of data for every 200 events were used as well as the grouped total of 1,000 events.

Results

Dual effect of octopamine in transmitter release: excitatory and inhibitory

In our experiments we consistently found in both crabs and crayfish that OA sometimes increased, and other times decreased the amplitude of excitatory postsynaptic currents (EPSCs) and excitatory postsynaptic potentials (EPSPs). Threshold concentration for this dual effect of OA was 10^{-8} mol l^{-1}. For the analysis, we normally used 10^{-6} mol l^{-1} OA. The dual effect of OA is shown qualitatively in Fig. 1 for averaged EPSC recordings in the crab (A) and EPSP recordings in the crayfish (B). In the crab, the percentage of excitation and inhibition was

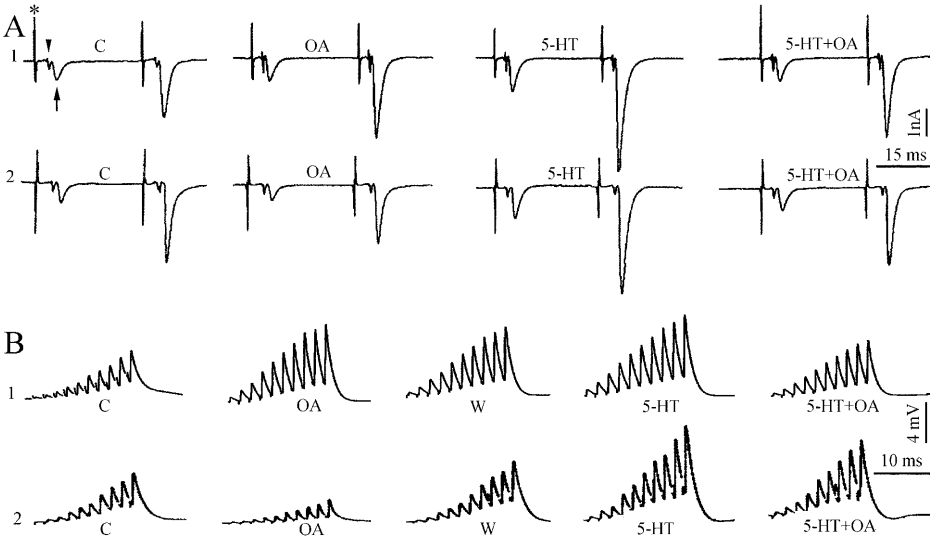

Fig. 1 Effect of octopamine ,OA (10^{-6} mol l^{-1}), serotonin, 5-HT (10^{-8} mol l^{-1}) and cocktails of the two on **A** excitatory postsynaptic currents (EPSCs) (A) and **B** excitatory postsynaptic potentials (EPSPs). The EPSCs were elicited by the twin pulses to the slow closer excitor (SCE) of the crab, the EPSPs by short trains of 40-Hz stimulation of the opener excitor in the crayfish. 300 samples were averaged for each EPSC trace. Details on the time of sampling in Fig. 2. *Upper traces* (*1*) in **A** and **B**: excitatory effects of OA, *in the EPSC records best seen in the second response to the twin pulses. Lower* traces (*2*) in **A** and **B**: inhibitory effects of OA. The EPSC records shown in **A1** depict the stimulus artifact (*asterisk*), followed by a depolarization (*arrowhead*) of the motor axon under the macropatch electrode (nerve terminal potential) which is then followed by the EPSC (*arrow*)

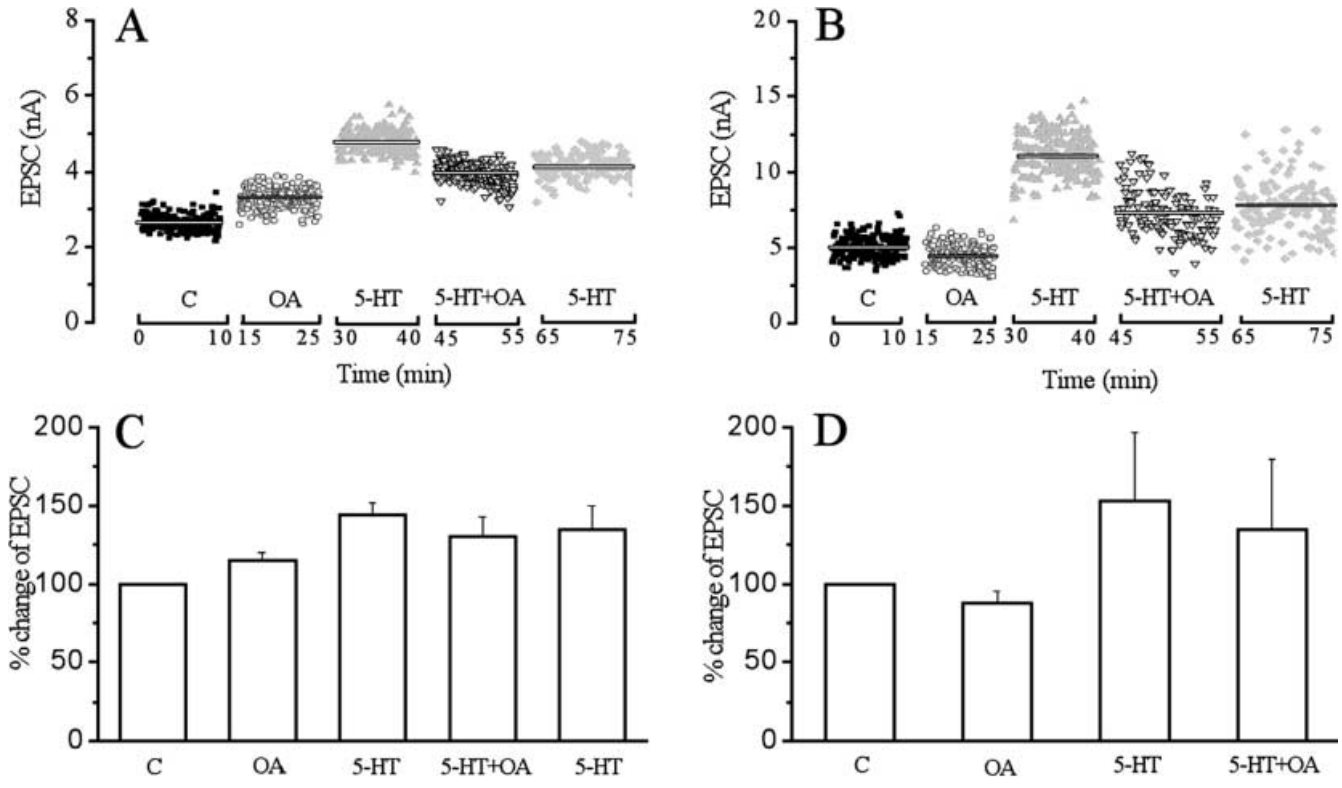

Fig. 2A–D Effect of OA (10^{-6} mol l^{-1}), 5-HT (10^{-8} mol l^{-1}) and cocktails of the two on EPSCs elicited by the second of twin pulses to SCE at 0.5 Hz stimulation for 10 min in the crab. **A**, **B** Individual experiments with excitatory or inhibitory effects of OA. After each 10-min recording sequence, the superfusion was changed to the new composition. The recording was resumed after 5 min of equilibration. **C**, **D** Effects normalized to controls (*c*) in six and eight experiments, respectively. The *bars* indicate mean ± SEM. Both effects of OA are statistically significant ($P < 0.01$)

about 50:50. In the crayfish, the excitatory effects were more commonly observed. Whether the effect of OA was excitatory or inhibitory clearly depended on intrinsic conditions of the animals because in cases when several preparations were obtained from the same individual, the responses were alike.

The two effects on EPSCs at the crab NMJ are quantitatively shown in Fig. 2 (parts A and B show examples of two typical individual experiments, parts C and D summarize several experiments with the effects related to normalized controls before application of the modulators). In the experiment shown in Fig. 2A, 10^{-6} mol l^{-1} OA enhanced the average amplitude of the EPSCs from 2.6 ± 0.2 nA in the control to 3.3 ± 0.3 nA. On the average, the excitatory effect of OA on EPSC amplitudes amounted to 14.3 ± 2.4% ($n = 6$, Fig. 2C). In the example shown in Fig. 2B, 10^{-6} mol l^{-1} OA reduced the amplitude of EPSCs from 5.0 ± 0.7 nA to 4.4 ± 0.7 nA. On the average, the inhibition was 12.7 ± 2.9% of the controls ($n = 8$, Fig. 2D). Both effects of OA are statistically significant ($P < 0.01$).

Identical results were obtained for the FCE axon in the crab. OA either increased (by 14.6 ± 5.5%, $n = 4$) or, in other preparations, decreased (by 11.6 ± 0.5%, $n = 2$) the amplitudes of EPSCs. A plot of the percentage changes in EPSC amplitudes of all experiments in the crab ($n = 20$) in a histogram clearly shows that the inhibitory and excitatory effects of OA represent two distinct modes of activity of OA (Fig. 3).

Whether the action of OA is excitatory or inhibitory seems to be consistent within a given animal. In animals where several leg preparations have been taken from within short succession, all preparations showed similar effects of OA. In six crabs all leg preparations exhibited excitatory OA effects, in three other animals, the effects were consistently inhibitory. Similar observations were made in the crayfish.

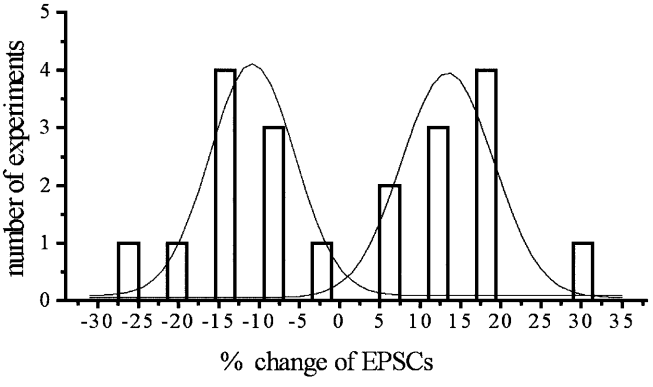

Fig. 3 Histogram of percentage changes in mean EPSC amplitudes in 20 crab preparations upon application of OA (10^{-6} mol l^{-1}). Positive values: excitation; negative values: inhibition. The curve represents Gaussian distribution

Effects of 5-HT

In all experiments, application of serotonin (5-HT, 10^{-8} mol l^{-1} to 10^{-7} mol l^{-1}) resulted in a significant enhancement of transmitter release in both preparations (seen in Figs. 1, 2, and 4), an effect which is in accord with a number of previous investigations in crustaceans (Dudel 1965; Kravitz et al. 1980; Enyeart 1981; Glusman and Kravitz 1982; Fischer and Florey 1983; Harris-Warrick and Kravitz 1984; Dixon and Atwood 1985, 1989a; Delaney et al. 1991; Qian and Delaney 1993; Wang and Zucker 1998). In the crab, the amplitudes of EPSCs generated by SCE were increased by about 50% ($n = 13$). The potentiation was reversible only after prolonged washing (minimum 30 min). The potentiating effect of 5-HT was also present after prior treatment of the preparations with OA and subsequent washing for 5 min, regardless of whether OA itself had an excitatory or an inhibitory effect (Fig. 2). In crab preparations, where prior exposure to OA resulted in an excitatory effect (Fig. 2A, C), application of 5-HT (10^{-7} mol l^{-1}) increased the EPSC amplitudes further, on average by $44 \pm 7.1\%$ ($n = 6$) of the controls in saline. In preparations with an initial inhibitory effect of OA, application of 5-HT (10^{-7} mol l^{-1}) increased the EPSC amplitudes by $55.6 \pm 20.5\%$ ($n = 4$, Fig. 2D). The individual experiment shown in Fig. 2B shows more than doubling of the EPSC amplitudes by 5-HT after an initial depression by OA (11.1 ± 1.4 nA against 5 ± 0.7 in the control).

Effects of cocktails of OA and 5-HT

In both preparations, cocktails of OA (10^{-6} mol l^{-1}) and 5-HT (10^{-8} mol l^{-1} to 10^{-7} mol l^{-1}) always reduced the potentiating effects of 5-HT alone applied in the same concentration as in the cocktail (Figs. 2, 4). In the crab, the reduction of the 5-HT potentiation by subsequent application of a cocktail was on the average $18.3 \pm 2.6\%$ ($n = 10$). However, when compared with the controls in saline, the cocktail still caused an increase of the amplitudes of EPSCs by approx. 30% ($n = 11$). Similar effects were also seen in most of the EPSP recordings from crayfish. The dampening effect of the cocktail on the 5-HT effect was irrespective of whether there was a prior exposure to OA and whether this had caused excitation or inhibition (Fig. 2).

In the crab, the inhibitory effect of a cocktail on the potentiating effect of 5-HT alone was independent of the sequence of application of the cocktail and the prior history of the preparation. Figure 5 gives a typical example of the effect of a cocktail of 10^{-7} mol l^{-1} 5-HT and 5×10^{-7} mol l^{-1} OA with an application scheme reversed from that shown in Fig. 4, i.e. the cocktail being applied first. The cocktail increased the EPSC amplitudes from 3.2 ± 0.8 nA in the controls to 7.1 ± 1.3 nA. The effect was abolished by washing for 30 min. Subsequent application of 10^{-7} mol l^{-1} 5-HT alone increased the amplitudes further to 8.8 ± 1.4 nA. Again, this effect

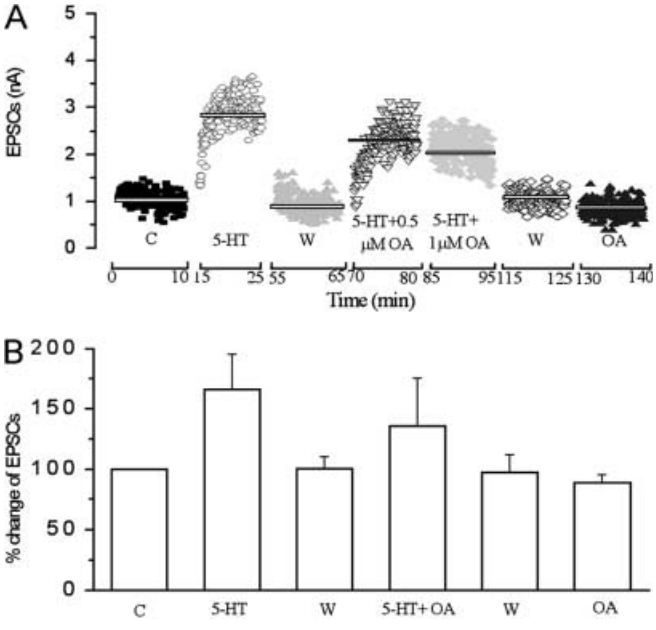

Fig. 4A, B Effect of 5-HT (10^{-8} mol l^{-1}) and cocktails of 5-HT (10^{-8} mol l^{-1}) plus OA at different concentrations, and OA (10^{-6} mol l^{-1}) alone on EPSCs in the crab. **A** Documentation of a representative experiment. The SCE axon was stimulated with twin pulses for 10 min in each trial, the amplitudes of the EPSCs to the second pulse are plotted. A period of 5 min was allowed for equilibration to each amine containing solution before resuming stimulation. When testing the reversibility of the effects (indicated by *W*), the preparation was washed for 30 min and 20 min, respectively, before recording. The inhibitory effect on the 5-HT induced potentiation was stronger with the higher OA concentration. The *bars* represent mean EPSC amplitudes. The time protocol is indicated at the baseline. **B** Effects of 5-HT and a cocktail of 5-HT (10^{-8} mol l^{-1}) plus OA (10^{-6} mol l^{-1}) normalized to the controls (C) in three preparations

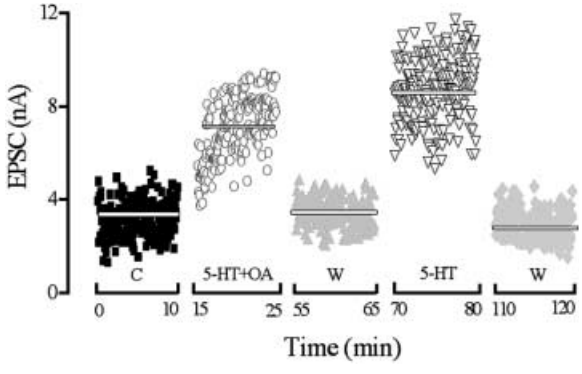

Fig. 5 Effect of a cocktail of 5-HT (10^{-7} mol l^{-1}) and OA (5×10^{-7} mol l^{-1}), and 5-HT (10^{-7} mol l^{-1}) alone on EPSC amplitudes of the crab. Details of stimulation, recording and washing were the same as indicated in the legend to Fig. 4. The time protocol is shown at the baseline

was reversible by washing for 30 min (final amplitudes 2.9 ± 0.6 nA, Fig. 5).

Since the overall amplitudes of the EPSPs are altered by OA and 5-HT, it was of interest to know if there were differences in short-term facilitation during the exposure

to the neuromodulators. The facilitation index (FI), obtained from the ratio of the tenth and fifth EPSP amplitudes, indicates that 5-HT, OA and the cocktail mixture did not have a significant effect on the magnitude of facilitation when compared to the control. The FI will show a reduction when the initial EPSPs show a greater enhancement than the subsequent EPSPs within a train, as is the case when a high concentration of 5-HT (10^{-7} mol l^{-1}) is applied to the preparation.

OA and 5-HT action on quantal parameters: *m*, *n*, and *p*

The responses measured in the muscle fibers have revealed alterations in synaptic transmission, but the cause of these changes may well be a combination of effects from pre- and postsynaptic components. Although it is assumed that most of the effects of either OA or 5-HT on EPSPs when applied individually are presynaptic (Dudel 1965; Breen and Atwood 1983; Florey and Rathmayer 1978; Fisher and Florey 1983; Jorge-Rivera et al. 1998; Beaumont and Zucker 2000; Southard et al. 2000), the effects of a cocktail mixture on release have not been addressed previously. In addition, OA actions on quantal parameters have not been investigated at all. The quantal evoked events within the crayfish preparation and with the response to the first of the twin pulses to SCE in the crab preparation are of such low output that individual evoked quantal events can be counted. This allows utilization of the direct method of determining mean quantal content (*m*).

Two commonly used stochastic procedures to fit the observed quantal counts to particular distributions were utilized in order to best estimate the number of active sites, *n* and the probability of release, *p* (Zucker 1973; Cooper et al. 1995). The results of both procedures are shown in Tables 1, 2. In the present study, *m* was obtained by direct counts of quantal events indicating that the AIC (Akaike Information Criterion and bootstrap) method provides a closer approximation for *m* by the product of the estimated *n* and *p* than the Zucker approach. It should be noted that the measures of *n* and *p* are only statistical descriptors and that links with synaptic morphology are not yet available for any synapse.

OA (10^{-6} mol l^{-1}) resulted in both an increase and a decrease in the mean number of quanta released (Table 1). In the crab, the increase of *m* averaged $23 \pm 9\%$ ($n=4$), the decrease $26 \pm 4\%$ ($n=4$). Exposure to 5-HT always increased *m* as compared to the saline controls by $42 \pm 14\%$ (results from crayfish, $n=9$, Fig. 6). Washing the preparations for 20 min was not effective in regaining the control values of *m* in most crayfish preparations (Fig. 6A). Subsequent application of a cocktail of 5-HT (10^{-8} mol l^{-1}) and OA (10^{-6} mol l^{-1}) resulted again in an increase of *m* by $43 \pm 37\%$ ($n=5$) when compared with the control before 5-HT application, but this increase was smaller than the increase in 5-HT (10^{-8} mol l^{-1}) alone ($53 \pm 25\%$, $n=5$); the difference is significant ($P=0.01$). Application of the cocktail to preparations in the presence of 10^{-8} mol l^{-1} 5-HT produced a statistically significant reduction by $22 \pm 7\%$ ($n=4$, $P=0.02$) in synaptic efficacy when compared to the effect of 5-HT alone (Fig. 6B, Table 2). The reduction of *m* is primarily a result of a reduction in the probability of release *p* as indicated in Tables 1 and 2.

The results obtained for the mean quantal content from the two preparations are in agreement with those described above and prove that the observed effects with the EPSCs and EPSPs are due to presynaptic effects of the modulators on transmitter release.

Table 1 Inhibitory (experiments 1–4) and excitatory effects (experiments 5–8) of octopamine, OA (10^{-6} mol l^{-1}) on quantal release by the slow closer excitor (SCE) in the crab. Two methods were employed: Zucker (1973) and AIC (Akaike information criterion and bootstrap). For details see Materials and methods. The values of *m* were the same when calculated by the two methods

Experiment		Counts	Zucker		AIC	
		m	*p*	*n*	*p*	*n*
1	Control	1.9	0.51	4	0.44	4
	OA	1.4	0.4	4	0.32	4
2	Control	1.4	0.45	3	0.35	4
	OA	0.9	0.31	3	0.22	4
3	Control	0.7	0.61	1	0.35	2
	OA	0.52	0.49	1	0.26	2
4	Control	0.95	0.8	1	0.47	2
	OA	0.79	0.7	1	0.35	2
5	Control	1.0	0.63	2	0.37	3
	OA	1.16	0.65	2	0.38	3
6	Control	0.26	0.15	2	0.13	2
	OA	0.5	0.34	2	0.25	2
7	Control	0.85	0.82	1	0.43	2
	OA	0.94	0.93	1	0.47	2
8	Control	1.2	0.67	2	0.4	3
	OA	1.7	0.81	2	0.56	3

Table 2 Effect of serotonin, 5-HT (10^{-8} mol l^{-1}) and a cocktail of 5-HT (10^{-8} mol l^{-1}) and OA (10^{-6} mol l^{-1}) on quantal release by the opener excitor in the crayfish (Zucker 1973; AIC). For details see Materials and methods. The values of *m* were the same when calculated by the two methods

Experiment		Counts	Zucker		AIC	
		m	*p*	*n*	*p*	*n*
1	Control	0.029	0.026	1	0.027	1
	5-HT	0.04	0.03	1	0.031	1
	5-HT/OA	0.024	0.023	1	0.024	1
2	Control	0.081	0.055	1	0.045	2
	5-HT	0.105	0.049	2	0.048	2
	5-HT/OA	0.08	0.079	1	0.08	1
3	Control	0.039	0.034	1	0.035	1
	5-HT	0.055	0.05	1	0.051	1
	5-HT/OA	0.041	0.038	1	0.039	1
4	Control	0.057	0.056	1	0.057	1
	5-HT	0.063	0.03	2	0.032	2
	5-HT/OA	0.053	0.049	1	0.049	1

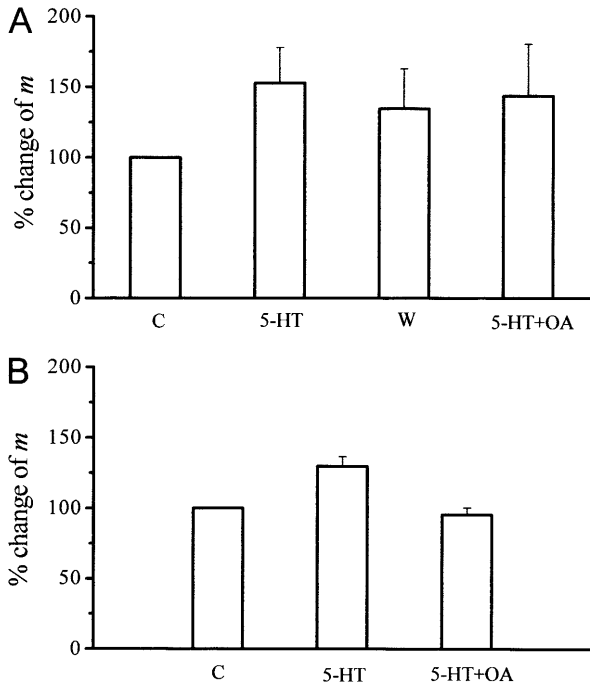

Fig. 6A, B Effect of 5-HT (10^{-8} mol l^{-1}) and a cocktail of 5-HT (10^{-8} mol l^{-1}) and OA (10^{-6} mol l^{-1}) on mean quantal content *m* in the crayfish preparation with different application schemes, normalized to controls (*c*) of five and four experiments presented in **A** and **B**. **A** Application of the cocktail after 20 min of washing produced a potentiation which was weaker than by 5-HT alone. **B** In preparations bathed in 10^{-8} mol l^{-1} 5-HT when applying the cocktail, the reduction of the potentiation of mean quantal content *m* by 5-HT through the cocktail was on average 22%

Discussion

In crustaceans, OA and 5-HT are present in identified neurons and in peripheral neurohaemal sites in close contact with the circulation (Cooke and Goldstone 1970; Evans et al. 1976; Livingston et al. 1981; Beltz and Kravitz 1986; Schneider et al. 1993; Thompson et al. 1994; Christie et al. 1997; Beltz 1999). These two amines, as well as numerous other modulators, are released together within the central nervous system and also circulate together throughout the haemolymph bathing peripheral targets such as the NMJs. This poses a problem in defining the role of individual modulators in relation to behavior, as has been the practice in the past. One must consider the actions of combinations of neuromodulators if one is trying to understand the complex control of synaptic efficacy in the animal and its importance for shaping behavior. In this study, we investigated the actions of cocktails of two of the most commonly studied amine neuromodulators, 5-HT and OA. Analyzing these effects at peripheral neuromuscular targets should permit the deduction of the role of combinations of modulators on synaptic processes within the central nervous system.

OA antagonizes the excitatory effect of 5-HT

This study reports novel findings in crayfish and crab that a cocktail of 5-HT and OA results in a reduction of synaptic efficacy compared with the effects of 5-HT alone. Our studies suggest that OA antagonizes the presynaptic action of 5-HT, and that the level of OA in the haemolymph could determine the magnitude of the potentiating effect of 5-HT on transmitter release. A parallel of this role of OA in reducing 5-HT effects seems to be present within the central nervous system. In the lobster, OA has been shown to exert inhibitory control on two pairs of serotonergic neurons which have projections both within the central nervous system and into peripheral neurohaemal organs (Ma and Weiger 1993). This inhibitory effect of OA on 5-HT release within the central nervous system should add synergistically to the inhibition exerted by OA on 5-HT effects we have described for the NMJ. Also for the crayfish escape response, opposite effects of OA and 5-HT on central neurons have been found (Glanzman and Krasne 1983). However, in this case 5-HT depressed responsiveness of the lateral giant axon, whereas OA enhanced it.

Since we observed identical opposing effects of the two modulators when present together in two different crustacean species, at the junctions of three different motor neurons belonging to different physiological types (slow and fast), and at two different (antagonistic) muscles, this mode of peripherally regulating the efficacy of motoneuronal patterns could apply more generally.

OA can enhance or reduce transmitter release

In addition, we report the novel finding that OA by itself does not always result in the well described enhancement of evoked transmitter release. In almost 50% of the experiments, a reduction of transmitter release in the presence of OA was observed. It has previously been assumed from studies on a number of different crustacean species including the crayfish *Astacus leptodactylus* that, if OA had an effect on EPSP amplitudes at neuromuscular junctions, this effect was always manifest in an increase (Florey and Rathmayer 1978; Kravitz et al. 1980; Breen and Atwood 1983; Fischer and Florey 1983; 1987; Harris-Warrick and Kravitz 1984; Jorge-Rivera et al. 1998). The only exception is a statement by Fischer and Florey (1983; p 190) based on experiments on the opener muscle in the crayfish, *A. leptodactylus*: "At higher concentrations, OA sometimes caused a depression of EPSP amplitudes and generally did not enhance EPSPs beyond the effects seen at the low concentration of 5×10^{-8} mol l^{-1}". Studies on *Drosophila* have recently shown that OA can reduce transmitter release also at insect neuromuscular junctions (Nishikawa and Kikodoro 1999). It should be added that OA, beside the presynaptic effects on transmitter release, exerts multiple postsynaptic effects on the level of the muscle fibers (e.g., Blau and Wegener 1994; Walther and Zittlau 1998).

Importance of the in vivo situation for in vitro experiments

Since the excitatory and inhibitory effects of OA were consistent in a given animal, we assume that intrinsic conditions create a "history" of the preparations in vivo which can determine the type and range of modulator effects studied in vitro. The inhibitory effect of OA on transmitter release might have occurred only in preparations obtained from animals the "history" of which was determined by high levels of endogenous 5-HT. There appears to be a positive link of social status, or aggressiveness in agonistic behavior among crustaceans with high levels of 5-HT (Yeh et al. 1996; Huber et al. 1997; Kravitz 2000; Sneddon et al. 2000). Injections of 5-HT increase aggression in subordinate individuals or elicit the dominant posture typical of aggressive animals; injections of OA, however, elicit submissive postures (Livingston et al. 1980; Antonsen and Paul 1997; Huber et al. 1997). If, in agonistic encounters of shore crabs, the OA concentration in a loser is high, he behaves submissively and the fighting is of low intensity and short, but if his OA concentration is low, the contest escalates because the encounter is prolonged and fighting behavior of the winner depends on the strategy the loser adopts (Sneddon et al. 2000).

The potential mechanisms underlying the 5-HT actions through second messenger systems have been investigated in crustacean motor axons (Enyeart 1981; Dixon and Atwood 1989a, 1989b; Goy and Kravitz 1989; Beaumont and Zucker 2000). The initial action of 5-HT is thought to be mediated upon binding to a G_S protein-coupled receptor by IP_3 and the prolonged effects via cAMP. Activation of either of these two systems can produce a wide range of other cellular cascades, from release of calcium from internal stores to modification of presynaptic ion channels (Beaumont and Zucker 2000) both leading to an enhancement of synaptic transmission. Interestingly, it has been established in a number of invertebrate systems that OA mediates its actions through cAMP in various tissues (Battelle and Kravitz 1978; Evans 1984; Groome and Watson 1989; Thompson et al. 1990). It is thus likely, considering our physiological results, that activation of the cAMP-mediated processes from OA stimulation can dampen the IP_3-mediated responses induced by the rapid actions of 5-HT. The long term action of 5-HT mediated through cAMP (Dixon and Atwood 1989b; Beaumont and Zucker 2000) may also be reduced if the same downstream actions are already activated by the prior presence of OA and a complete recovery has not been allowed to occur.

Much work remains to be done to determine which intracellular messenger systems and proteins may be activated or suppressed in the presence of just OA or 5-HT alone. This may foster the understanding of the antagonistic action of OA to 5-HT as we have reported when the two modulators are present together. We should not forget that there are numerous additional modulator players present acting in concert to influence synaptic functions, both within the central nervous system and at peripheral targets. Future studies of several neuromodulators in combination will greatly increase the complexity we already see in the aminergic and peptidergic modulation of neuronal properties and intercellular communication (Rathmayer 2001).

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